

The relationship between *Sarracenia oreophila* and an endophytic *Burkholderia*

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The relationship between *Sarracenia oreophila* and an endophytic *Burkholderia*

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LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance

ARA: Acetylene reduction assay

Bcc: *Burkholderia cepacia* complex

BLK: Bruhn, Lenke, and Knackmuss medium(Bruhn, Lenke et al. 1987)

BLKN: BLK with nitrogen added

CFU (colony forming units)

GFP: green florescent protein

LB: Luria-Bertani medium(Bertani 1951)

LSD: Least Significant Difference

LPS: lipopolysaccharide

LRC: Lateral root cracks

PGPB: Plant growth-promoting bacteria

TSA: Tryptic Soy Agar

SUMMARY

Plant growth-promoting bacteria (PGPB) have been studied in many agriculturally interesting plants, but never in pitcher plants.

Sarracenia oreophila (the green pitcher plant) is an endangered species in Georgia, Alabama, and North Carolina (Rice 2010). With the help of Dr. Jim Spain's lab, a previous student in Dr. Gerald Pullman's lab discovered evidence that nitrogen-fixing bacteria (*Burkholderia spp.*) live within these pitcher plants. This study aims to determine whether these nitrogen-fixing bacteria confer a benefit to their host plants by providing fixed nitrogen.

To do this, pitcher plants were inoculated with the *Burkholderia* and grown on a control medium, a medium without sugar (as the sugar causes the bacteria to grow until they hinder the plants), various media that are missing nitrogen-containing compounds usually provided in growth media, and a medium completely lacking nitrogen. These plants were compared to control plants on the same media that had not been inoculated with *Burkholderia*. The plants' biomass and root growth were measured.

The data suggest that *Burkholderia* may stimulate plant biomass growth when sufficient nitrogen is present and there may be a nitrogen-threshold that needs to be met in order to sustain the *Burkholderia-Sarracenia* symbiosis. Also, the *Burkholderia* has a negative effect on roots grown in high-nitrogen media, possibly due to competition for nutrients.

CHAPTER 1: BACKGROUND

1.1 Introduction

Plant Growth Promoting Bacteria and their symbioses with plants

Plant growth-promoting bacteria (PGPB) have potential agricultural and phytoremediation applications. PGPB can increase the growth of plants by reducing pathogens, other biotic stress, or abiotic stress (Compant, Clement et al. 2010). PGPB could be of great ecological and agricultural importance, if they could be reliably used in place of chemical fertilizers and pesticides without being pathogenic to plants.

It was once thought that healthy plants are completely devoid of bacteria, but now we know that plants play host to numerous bacteria, mostly in the rhizosphere (Smith 1911). The rhizosphere is made up of the soil directly around the roots of a plant. PGPB can also be found living within the tissues of plants. When this occurs, the PGPB are called endophytes or endophytic bacteria. Common PGPB include species of *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Azotobacter* (Lodewyckx, Vangronsveld et al. 2002; Mastretta, Barac et al. 2006).

Thus far, applications of PGPB in the field have largely failed to produce the desired results (Compant, Clement et al. 2010). An inability to become rhizosphere competent is often cited as the most logical reason (Lugtenberg, Dekkers et al. 2001). For a bacterium to be rhizosphere competent, it needs to be capable of becoming a member of the community surrounding the roots of the plant.

De Weert *et al.* linked root exudation and chemotaxis to colonization when they removed a chemotaxis gene from an growth-promoting strain of *P. fluorescens* and the bacteria could no longer be found in the roots of the plant (Weert, Vermieren et al. 2002). Colonization may also be affected by factors such as ability to synthesize amino acids or vitamins that the plant needs, ability to synthesize NADH hydrogenase, LPS,

fimbriae, flagella, type IV pili, twitching motility, and cell wall degrading enzymes (like cellulases and polygalacturonases) (Coenye and Vandamme 2003). Colonization has been shown to occur through lateral roots cracks (LRC) that form as new roots grow (Gough, Vasse et al. 1997; Coenye and Vandamme 2003; Goormachtig, Capoen et al. 2004; Compant, Reiter et al. 2005). From there, the bacteria can travel to other parts of the plant through the xylem (Coenye and Vandamme 2003). Distinct collections of bacteria have been found in the rhizosphere, roots, stems, leaves, flowers, fruits, and seeds of plants (Sessitsch, Reiter et al. 2002; Berg, Krechel et al. 2005; Okunishi, Sato et al. 2005). Berg *et al.* found bacteria in the aerial parts of potato plants that were not also present in the rhizosphere or roots, suggesting that the bacteria do not necessarily have to come from the roots (Berg, Krechel et al. 2005).

Some of the endophytes found in plants can also be human pathogens. The factors that help these pathogens infect humans may be the same factors that help them colonize plants (Berg and Hartmann 2005). For example, the O-antigen (an LPS protein known to be a virulence factor) has been shown to contribute to root colonization by aiding in adhesion (Dekkers, Bij et al. 1998). *Burkholderia* may be one such species (Compant, Nowak et al. 2008).

Some possible methods that PGPB may use to promote plant growth include nitrogen-fixation, production of siderophores that could help the plant take up iron, production of antibiotics that attack the plant's pathogens, production of lytic enzymes that also attack the plant's pathogens (Coenye and Vandamme 2003), and by triggering induced systemic responses (a plant immune system) (Compant, Duffy et al. 2005). Antibiotic production, nitrogen-fixation, and plant colonization may all be controlled via quorum-sensing (Soto, Sanjuan et al. 2006; Latour, Faure et al. 2008).

Carnivorous Plants

Carnivorous plants were discovered by Darwin (Darwin 1875) in 1875. These plants subsist on nutrients from the soil and from prey which the plants capture through a variety of means including pressure-sensitive traps, slippery pitchers, and sticky mucus. They attract their prey with color and nectar (Lloyd 1942). Pollen and foliar leachate from the canopy can also fall into or onto the traps and provide added nutrition (Juniper, Robins et al. 1989). There are 600 species of carnivorous plants in 9 families and 6 orders (Schlauer 1986; Ellison 2006). Carnivory has evolved six (Takhtajan 1969) or seven (Albert, Williams et al. 1992) times.

The majority of carnivorous plants grow in bogs and fens (Adamec 1997). These locations can be hostile due to low soil pH, water-logged and poorly-aerated soil (Armstrong 1979), and low nutrients (Adamec 1997). Carnivory is only one adaptation strategy for these conditions (Juniper, Robins et al. 1989).

A plant must meet the following criteria in order to be considered carnivorous:

- Catch prey (Adamec 1997)
- Absorb metabolites from prey (Adamec 1997)
- Use the absorbed metabolites for growth (Lloyd 1942)
- Grow in sunny, nutrient-poor environments (Juniper, Robins et al. 1989)
- Poor competitors that can only occur in extreme environments (Gibson 1983; Ellison 2006)
- Tolerant to waterlogging and low-temperature fire (Dixon, Pate et al. 1980)
- Tolerant to heavy metals (Greger 1999)

Different species of carnivorous plants have different levels of dependence on the nutrients they acquire from prey, ranging from total dependence to total independence (Adlassnig, Peroutka et al. 2005). For most species, carnivory is not

indispensable. In several species, nutrient uptake through the leaves can stimulate additional uptake through the roots (Harder and Zemlin 1967; Aldenius, Carlsson et al. 1983; Adamec 1997; Adamec 2002). The amount of nutrients that carnivorous plants can absorb from their leaves versus the soil also varies a lot between species (Adlassnig, Peroutka et al. 2005). The research that has been done on the subject of how much nutrition comes from which sources have been spotty, yielding ambiguous results. For example, Ellison and Gotelli (Ellison and Gotelli 2001) reported that prey accounts for 10-90% of the nitrogen budget of *Sarracenia purpurea*. Another study, done by Wakefield (Wakefield, Gotelli et al. 2005) showed no increase in plant success after addition of prey.

Carnivorous plants can survive in very low-nutrient conditions. They have adapted to grow slowly, utilize resources efficiently, and reuse resources from older parts of the plant (Dixon, Pate et al. 1980). They have particularly high efficiency in reusing nitrogen and phosphorous from older parts in order to produce new growth (Karlsson 1988). One paper reports that 65% of *Sarracenia purpurea*'s nitrogen budget comes from nitrogen that was stored during previous years (Butler and Ellison 2007). The leaves of carnivorous plants have much lower concentrations of nitrogen, phosphorous, and potassium than those of non-carnivorous plants (Ellison 2006). These concentrations are low enough to be considered below the limit for growth in non-carnivorous plants. Christensen (Christensen 1976) grew *Sarracenia flava* in a completely nutrient-free medium for four and a half months as part of an experiment, and the plants did not die. Carnivorous plants have adapted to be so efficient with low levels of nutrients that high-nutrient conditions can actually be fatal (Eleuterius and Jones 1969). Carnivory increased the growth of plants, but the effect decreases and plateaus as nutrients in the soil are increased (Ellison 2006).

Since carnivorous plants can survive such harsh conditions, they are good competitors in harsh environments. However, when pitted against non-carnivorous plants in hospitable environments, their slow growth and relative photosynthetic inefficiency (only 20-50% that of non-carnivorous plants) make them poor competitors (Gibson 1983; Ellison 2006). Carnivorous plants are so photosynthetically inefficient compared to non-carnivorous plants because they spend their resources on making things like mucilage, leaf hairs, and traps (Ellison 2006).

Researchers used to assume that carnivorous plants have weak root systems, but Adlassnig *et al.* (Adlassnig, Peroutka et al. 2005) compiled data from various studies to show that there is a wide variety of root systems within the various families of carnivorous plants. Carnivorous plants have been shown to have no association with mycorrhizal fungi (MacDougal 1899). *Roridula* is the only exception. Plant roots fulfill four basic functions: anchorage, water uptake, nutrient uptake, and nutrient storage. In carnivorous plants, some of these functions are taken over, in part, by the leaves.



Figure 1: Various carnivorous plants. Clockwise from top right: *Nepenthes* species, Venus Flytrap (*Dionaea muscipula*), white-top pitcher plant (*Sarracenia leucophylla*), and *Sarracenia* species. These photos were taken at the Atlanta Botanical Gardens.

Sarracenia oreophila

There are eight species within the genus *Sarracenia* (Adlassnig, Peroutka et al. 2005). *Sarracenia oreophila* (Kearney) Wherry (Wherry 1933), the green pitcher plant, is a federally endangered species found in northern Georgia, northern Alabama, and western North Carolina (Carter, Boyer et al. 2006). As of 2006, there were only 35 known populations, 32 of which were to be found in Alabama (Carter, Boyer et al. 2006). This species is endangered due mainly to habitat destruction and illegal collection by hobbyists (Troup and McDaniel 1980). The few habitats in which they remain are protected and well-maintained (Rice 2010).

S. oreophila is usually found in moist, sloping, sandy areas in bogs or on riverbanks (Schnell 1980; Folkerts 1982; Patrick, Allison et al. 1995; Carter, Boyer et al. 2006).

In appearance they are very similar to their close relative *S. flava*, but not as tall or hardy (Rice 2010). They are mostly green with variable amounts of purple streaks. Their flowers are said to have a characteristic cat urine odor (Schnell 1980).



Figure 2: *S. oreophila* growing at the Atlanta Botanical Gardens.



Figure 3: An unearthed *S. oreophila* plant. Note the underground organs. The roots are growing from a mass called a rhizome.

Burkholderia

Burkholderia are versatile microorganisms that inhabit a wide variety of ecological niches ranging from soil to the human respiratory tract (Coenye and Vandamme 2003). The genus contains 30 species, but much of the literature focuses on the *Burkholderia cepacia* complex (Bcc), a collection of at least nine related *Burkholderia* species (Luvizotto, Marcon et al. 2010) that can be found in soil, water, plants, animals, hospitals, and the lungs of Cystic Fibrosis patients (Coenye and Vandamme 2003). The

genus *Burkholderia* was created by Yabuuchi (Yabuuchi, Kosako et al. 1992) and named after Burkholder who had discovered the genus but classified it as *Pseudomonas* (Burkholder 1950).

There are two species of *Burkholderia* that are well-known animal pathogens. *B. mallei* causes a disease called glanders in horses that can be passed to humans. It was used by Germany as a biological weapon in World War I (Wheelis 1998). *B. pseudomallei* causes a disease called melioidosis in humans (Dance 1991). All nine species in the *Burkholderia cepacia* complex have been found in the sputum of Cystic Fibrosis patients (Coenye and Vandamme 2003). Some strains of *B. cocovenenans* have been linked with cases of food poisoning and have been recovered from contaminated water and milk (Zhao, Qu et al. 1995).

Many *Burkholderia* have been shown to be plant pathogens (Smith 1911; Goto and Ohata 1956; Kurita and Tabei 1967; Ballard, Palleroni et al. 1970; Uematsu, Yoshimura et al. 1976; Palleroni 1984; Urakami, Ito-Yoshida et al. 1994), but most species are soil bacteria that have non-pathogenic effects on plants (Coenye and Vandamme 2003). Some have even been shown to have beneficial effects on plants (Parke, Rand et al. 1991; McLoughlin, Quinn et al. 1992; Bowers and Parke 1993; Hebbar, Martel et al. 1998; Van, Berge et al. 2000; Moulin, Munive et al. 2001; Compant, Reiter et al. 2005; Taghavi, Garafola et al. 2009). Even though these bacteria can be considered endosymbionts, all species that have been discovered can be grown on media in the lab (Coenye and Vandamme 2003).

Coenye and Vandamme (Coenye and Vandamme 2003) performed a meta-analysis of the literature in an attempt to find out whether the pathogenic *Burkholderia* and the environmental *Burkholderia* are the same. Their results were not clearly conclusive, but they decided that “human isolates are not necessarily distinct from environmental ones”.

Burkholderia are also promising tools for bioremediation (Kilbane, Chatterjee et al. 1982; Folsom, Chapman et al. 1990; Krumme, Timmis et al. 1993; Bhat, Tsuda et al. 1994; Shin and Spain 2009) and have been the source of novel antibiotics (Mori, Yamashita et al. 2007; Seyedsayamdost, Chandler et al. 2010).

1.2 Literature Review

Burkholderia have been found in a wide variety of environments including soil, water, plants, animals, hospitals, and human lungs (Coenye and Vandamme 2003). *Burkholderia* has long been known as a plant pathogen, with references dating back to the early 1900s (Coenye and Vandamme 2003). In the 1990s, studies started appearing that investigated the plant growth-promoting abilities of *Burkholderia* and other bacteria (Coenye and Vandamme 2003). Here we detail some of the studies done on *Burkholderia* and other plant growth-promoting bacteria (PGPB) and their effects on the plants they colonize.

One of the main reasons that PGPB-plant interactions are of interest is that they may be viable substitutes for expensive fertilizers that can be detrimental to the environment. Three approaches to use PGPB to provide fixed nitrogen to plants have been proposed: 1. Induce root nodule formation; 2. Identify nitrogen-fixing endophytes; 3. Incorporate nitrogen-fixing genes from PGPB directly into plants (Stoltzfus, So et al. 1997). Options 1 and 3 are both complicated because they involve altering the genome of the plant. Therefore, most studies have utilized option 2 and have tried to isolate endophytes that already interact with the target plant. If the endophytes isolated did not fix nitrogen, they could be engineered to do so. Inserting genes into bacteria is much simpler than inserting genes into plants.

No studies have been done to isolate endophytes living within the tissues of pitcher plants, but some have been done on bacteria living inside the pitcher traps.

Prankevicius *et al.* isolated three species of *Azotobacter* from the pitchers of *Sarracenia purpurea* (Prankevicius and Cameron 1989). All three species were found to fix nitrogen (by growth in nitrogen-free media and ARA) and occurred in higher concentration in the pitchers than in the surrounding environment, suggesting a positive non-random association. Since leaves do not always trap insects, the bacteria may be an alternative source of nutrition. One of the three species of bacteria was grown at 24°C and turned brown over time, which makes it sound like *Burkholderia*. All three isolated species were identified as *Azotobacter*, but the identification was only based on morphological characteristics (Prankevicius and Cameron 1989).

Butler and Ellison used ^{15}N supplied to pitchers and roots to track where *Sarracenia purpurea* (a relative of *Sarracenia oreophila*) gets its nitrogen. The pitchers assimilated 57% of the nitrogen supplied while roots only assimilated 2.5% (Butler and Ellison 2007). Together, these amounts only accounted for 33% of the pitcher plant's nitrogen budget. The other 67% is supposedly taken from the nitrogen stored from previous years (Butler and Ellison 2007). The paper makes no mention of nitrogen-fixing bacteria.

Albino *et al.* studied endophytes within a different species of carnivorous plant called *Drosera villosa*. These plants have sticky leaf traps. Albino *et al.* studied the number of CFU in the rhizosphere (soil directly around roots), endorhizosphere (within roots), phyllosphere (surface of leaves), and endophyllosphere (within leaves) of *Drosera*. Overall, there were more bacteria outside than inside, but the bacteria inside were more diverse (Albino, Saridakis *et al.* 2006). The more favorable environment inside the plant leads to more competition and therefore more diversity. Sixty three bacteria were isolated, including many nitrogen fixers, including *Burkholderia*.

Caper plants (*Capparis spinosa* L.) have an unusually high concentration of nitrogen (Andrade, Esteban *et al.* 1997), so seemed to be a good candidate for housing

nitrogen-fixing endophytes. Eleven nitrogen-fixing endophytes were isolated from the rhizosphere of these plants by plating diluted rhizosphere soil on nitrogen-free media (Andrade, Esteban et al. 1997). Tang *et al.* surface-sterilized nipa palm tree roots in bleach, and used 16S rRNA to identify the 58 endophytic bacterial species that came out (Tang, Hara et al. 2010). Of the 58 species, 21 were *Burkholderia*. Of the 21 *Burkholderia* species found, 11 were part of the Bcc. Of the 11 species in the Bcc, seven were shown to fix nitrogen via an acetylene reduction assay (ARA), showing that the bacteria reduce acetylene to ethylene, which is indicative of nitrogen-fixation. They also isolated a couple of *Azospirillum* species, known nitrogen-fixing endophytes in grasses (Kapulnik, Kigel et al. 1981).

Luvizotto *et al.* isolated 39 species of *Burkholderia* from the rhizosphere of sugarcane roots, most of which were part of the Bcc, which had already been shown to interact beneficially with sugarcane (Mendez, Pizzirani-Kleiner et al. 2007; Luvizotto, Marcon et al. 2010). These species were evaluated for ability to fix nitrogen, produce siderophores, solubilise inorganic phosphates, produce indole-acetic acid, and inhibit sugarcane pathogens. At least two of these features were found in each isolate.

Taghavi *et al.* isolated 78 endophytes from poplar trees, including members of the *Burkholderia cepacia* complex (Bcc). The endophytes were found to produce phytohormones and other molecules that aid plant growth. However, none of them fixed nitrogen. Cuttings inoculated with one of the isolates (*S. proteamaculans*) produced denser roots much faster than cuttings that were not inoculated (Taghavi, Garafola et al. 2009). This is one of many examples showing that endophytes do provide a benefit to the plants that they live within.

Stoltzfus *et al.* surface sterilized rice roots, ground them in a blender, and spread the result on bacterial growth media. A collection of 130 highly diverse endophytes was isolated, many of which were shown to fix nitrogen either by acetylene reduction assay

(ARA) or detection of *nifD* genes (persistence nitrogen-fixation genes) (Stoltzfus, So et al. 1997). Sterile rice seedlings were then inoculated with the bacteria that grew. After growing for 25 days, the plants were surface sterilized, ground in a blender, and the macerate was spread onto plates to verify the endophytic competence of the putative endophytes isolated. The results of the re-isolation were spotty, and the ones that were re-isolated were deemed to be more aggressive colonizers than the others (Stoltzfus, So et al. 1997). No observations were made about whether inoculated plants produced more biomass, which is a shame because it would have been a simple step to add.

Compant *et al.* inoculated grape seedlings with *Burkholderia* sp. Strain PsJN by transferring the plants into tubes of an agar medium that had been inoculated with the bacterium (Compant, Reiter et al. 2005). After growing for 15 days, the inoculated plants weighed more than those that had not been inoculated and had longer leaves (% growth). There was no difference in the lengths of the roots.

Peix *et al.* isolated a *Burkholderia cepacia* species (a member of the Bcc) from common bean plants. Non-sterile bean plants growing in soil were inoculated with the bacterium. The inoculated plants had 44% more phosphorous and nitrogen content than the plants that were not inoculated. The inoculated plants also had significantly greener leaves, indicating that the *Burkholderia* antagonizes a fungus (*Fusarium oxysporum*) that caused chlorosis (yellowing) in beans (Peix, Mateos et al. 2001). (Leaf quality was measured with the CIAT scale used by Pastor-Corrales and Abawi (Pastor-Corrales and Abawi 1987).)

It is important to understand how PCPB colonize the plants that they live in so that we can reproduce the phenomenon at high frequency for agricultural and conservational purposes.

Gough *et al.* (1997) used a PGPB with the *lacZ* gene to visualize where colonization takes place. Concentrated blue pigment resulting from the degradation of X-

gal on the growth medium was found around the lateral root cracks (LRC) or sites of lateral root emergence (Gough, Vasse et al. 1997). This is evidence that the LRC are the sites of colonization.

Compant *et al.* (2005) looked at the roots of *Burkholderia*-inoculated grape seedlings and saw yellow autofluorescence indicative of accumulation of phenolic compounds (Compant, Reiter et al. 2005). Phenolic compounds are indicative of a host response against cell wall-degrading enzymes like endoglucanase and endopolygalacturonase. The presence of these enzymes was then verified on KW (Kim-Wimpenny) medium (Kim and Wimpenny 1981). GFP-labeled bacteria were seen clumped around the sites of lateral root emergence. Another visible host response was the thickening of the cell walls on the outer layer of root cells. Bacteria can get through lateral root cracks (Compant, Reiter et al. 2005), root tips (Hurek, Reinhold-Hurek et al. 1994), or epidermal layer (Reinhold-Hurek and Hurek 1998).

Bordiec *et al.* (2011) showed that *Burkholderia* (a known grapevine endophyte) and *Pseudomonas* (a known pathogen) attach to the surface of grapevine cells using pili, flagella, and a type III secretion system (Bordiec, Paquis et al. 2011). They showed that *E. coli* (a benign negative control) did not attach to the cells. Both *Burkholderia* and *Pseudomonas* up-regulated plant defenses (production of reactive oxygen species, salicylic acid, and jasmonic acid and induction of MAP-kinase pathway), but *Pseudomonas* produced a hyperactive response, indicative of a plant defending itself against a pathogen. Before Bordiec, many papers had talked about induced systemic response after inoculation with bacteria, but few had talked about the local responses that happen immediately after application of bacteria.

To determine the path of colonization, Compant *et al.* (2005) separated the roots, nodes, and leaves of grape seedling inoculated with *Burkholderia* and ground them periodically over the course of 96 hours in order to determine what order the plant parts

were colonized in. Colonization was observed to take place in distinguishable stages: through the rhizosphere (soil around roots), inner root, node, and then leaves (Compant, Reiter et al. 2005).

CHAPTER 2: OBJECTIVES AND APPROACH

2.1 Objectives

Experiments investigating endophytic PGPB have never been done with pitcher plants, probably because most research involving PGPB is focused on increasing agricultural output. *Sarracenia oreophila* is not an agricultural plant, but it is worth studying for conservational purposes. There have been studies of the bacterial community living in the pitchers, but this is the first study involving bacteria that live within the plant's tissue.

The objective of this study is to investigate whether a species of *Burkholderia* isolated from the green pitcher plant (*Sarracenia oreophila* (Kearney) Wherry) confers a benefit to the plant. Since the bacterium fixes nitrogen and the plant lives in low-nitrogen environments (Juniper, Robins et al. 1989), we hypothesize that the bacterium may fix nitrogen in a manner that makes the nitrogen available to the plant.

Sterile green pitcher plants were grown with and without the *Burkholderia* on media with and without nitrogen. We hoped to show that the plants grown with the *Burkholderia* inoculum would have a greater increase in biomass on a nitrogen-free medium than the plants without the *Burkholderia*.

Study Goals:

- Determine the *Burkholderia*'s effect on biomass
- Determine the *Burkholderia*'s effect on root growth

This will involve the following steps:

- Developing appropriate media
- Inoculating sterile green pitcher plants with *Burkholderia*

- Measuring biomass and root growth periodically
- Re-isolating *Burkholderia* from inoculated plants
- Performing ANOVA analyses

2.2 Species Selection and *Burkholderia* isolation

S. oreophila Germination

The plants used in this study were remnants of a previous study carried out by Northcutt *et al.* (Northcutt, Davies et al. 2009). The material was provided by the Atlanta Botanical Gardens in the form of seed. The seeds were washed in running tap water for ten minutes, agitated in three milliliters of sulfuric acid for ten minutes, and agitated in sterile water for three periods of five minutes each (Uhnak 2003). The seeds were rinsed with the help of a sterile fine metal sieve lined with nylon (fabric 06400JP-72 with 162x162 fibers per inch from Decotex Inc, Pawling, New York) to reduce damaging the fragile acid-treated seeds.

The seeds were germinated on various concentrations of MS medium (Murashige and Skoog 1962), as they were part of a germination experiment. After the germination experiment had concluded, all plants were transferred to medium 2517 (a multiplication medium, see Table 1). All plants remained on 2517 for at least one month prior to induction into the new experiment.

Plants were kept at 25-26 °C under a 16-hour light/8-hour dark cycle supplied by cool, white fluorescent lamps with an intensity of 30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

S. oreophila Rhizome Bud Induction Experiments and *Burkholderia* isolation

Northcutt *et al.* attempted to induce growth of buds from slices of *S. oreophila* rhizome that were left over from a previous experiment (Northcutt, Davies et al. 2009). The rhizome originally came from the greenhouse at the Atlanta Botanical Gardens. The rhizomes were agitated in 10% Liquinox with 0.2% Tween 20 for ten minutes, rinsed in

running tap water for thirty minutes, surface sterilized in 20% hydrogen peroxide for ten minutes, and agitated in sterile water for five periods of five minutes each (MacKay, Becwar et al. 2006). The sterile rhizomes were then cut into 1/8 inch slices with a sterile scalpel. These slices were placed on various media and stored under cool, white fluorescent lamps. The induction experiment was unsuccessful, but an interesting surprise arose from the process. Bacteria began to grow out of the vascular tissue of some of the supposedly sterile rhizome slices.

Rhizome slices from the bud induction experiment were given to Professor Jim Spain for isolation and identification of bacteria. Professor Spain identified the bacterium as a species of *Burkholderia* and determined that it is capable of fixing nitrogen.

This bacteria was streaked on LB repeatedly to ensure the purity of the culture. Only one type of colony morphology was ever observed.

2.2 Inoculation Protocol

The sterile plants were taken off of the 2517 shoot multiplication medium they were being held on and placed on sterile disposable petri dishes for dissection. The 2517 medium encourages pitcher plants to multiply asexually. Since individual plants were desired for the experiment, the plants needed to be cut apart using a sterile scalpel. All scalpels and tweezers were sterilized with 70% EtOH and flame.

During dissection, the roots of the plants were removed. This was done so that all plants started with the same number of roots (zero) and so that the inoculum would have a fresh wound to enter through.

Plants were placed into individual sterile boxes (Magenta Corp., Chicago) containing 20ml of experimental medium. Twenty plants were placed on each medium (described in section 2.3). Half of these were dipped into a liquid suspension of *Burkholderia* before being placed into the boxes.

Liquid suspensions of *Burkholderia* were provided by Zohre Kurt of Professor Jim Spain's lab. She grew samples that were stored in 30% glycerol at -80° C in an endophyte medium described by Taghavi *et al.* (Taghavi, Garafola et al. 2009), a nitrogen-containing medium. She did this because even though the *Burkholderia* can fix nitrogen, it grows faster on nitrogen-containing media. *Burkholderia* is a slow-growing bacterium, but when supplied with nitrogen, it only takes about two days for it to reach the desired OD (0.13 - 0.23). She then washed the bacteria by centrifugation at 6000 rpm for 20 minutes twice using a nitrogen-free version of the endophyte medium from Taghavi *et al.* (Taghavi, Garafola et al. 2009), and resuspending them in the same nitrogen-free medium.

All plants were kept at 25-26 °C under an 16-hour light and 8-hour dark schedule supplied by cool, white fluorescent lamps and transferred to fresh media once per month.

In order to determine biomass and growth rate, boxes of media were weighed prior to being habited with plants. Due to the fact that the pitcher plants used were young and small, care had to be taken to make sure there were no discrepancies while measuring the weights of the boxes before and after habitation. Media lose weight quickly as water evaporates. One day is long enough to cause a 0.2g change. Therefore, all boxes were weighed on the day the plants were to be transferred. Also, to keep the plants sterile, it was necessary to wrap the lid of each box in parafilm (Pechiney Plastic Packaging, Chicago). An inch-wide piece of parafilm weighs about 0.2g, too much to be considered negligible. Therefore, each box was weighed along with its own labeled piece of parafilm.

Every time plants were transferred from one set of boxes to another, detailed observations of each plant were recorded. Roots were counted and measured. Root characteristics like color and degree of hairiness were recorded. Leaves were counted

and classified as green, brown, yellow, white, or dead. The part of the leaf any discoloration was found on was recorded (tip, middle, bottom). A photograph was taken of each plant and each plant's roots to document any and all visible changes.

Some of the times when plants were transferred, the media in the old boxes were checked for bacteria to make sure that bacteria was present in the inoculated boxes and not present in the clean boxes. This was done by using a sterile scoopula to excise a 1cm² piece of medium from the old, empty box and depositing it into a sterile vial containing 1ml of indexing medium (Bacto® Nutrient Broth, 0003-17-8, Difco, Detroit). Vials were kept at 25-26 °C in a cabinet for one week before being observed. Vials containing bacteria were visibly cloudy compared to control vials. Vials without bacteria appear the same as a control vials. The plants corresponding to vials that did not show the expected result were removed from the experiment.

All transfers were done in a sterile environment within a laminar flow hood that had been sprayed out with ethanol (SterilGARD Hood, Class II, Type A/B3, The Baker Company Inc.).

2.3 Media Design

In a precursor to this experiment, Kylie Bucalo of Professor Gerald Pullman's lab, grew *S. oreophila* plants on three different media: 2493, 2566, and 2567. Medium 2493 is a control medium that is essentially a 1/3 concentration MS (Murashige and Skoog 1962) (See Table 1). The Atlanta Botanical Gardens found this to be a viable medium for growing pitcher plants. Pitcher plants cannot grow on full MS because they are adapted to low nutrient environments (Juniper, Robins et al. 1989). The level of nutrients in full MS is enough to be toxic for these efficient plants.

Medium 2566 is the same as 2493 but with the NH_4NO_3 and KNO_3 removed. K_2SO_4 was added as a substitute potassium source. At the time, the investigators believed that removing the NH_4NO_3 and KNO_3 would be sufficient to test the hypothesis.

Medium 2567 is the same as 2566, but without the sucrose.

For this experiment, 2566 was eliminated because providing sugar in the medium proved to be too beneficial for the bacteria. Bacteria grew rapidly on this medium and choked out the plants. Thus, 2566 was replaced with 2633 which is simply the control medium (2493) without sucrose. The purpose of 2633 was to show that the pitcher plants can grow without sugar. This is a necessary control because the true nitrogen-free medium should not contain sugar so the bacteria do not choke out the plants.

After some deliberation, we decided to remove all sources of nitrogen from the medium, no matter how minute. It still contained B vitamins, glycine, and EDTA, all of which contain nitrogen. Several new media were developed.

Medium 2702 is the control medium (2493) without B vitamins or glycine. The purpose of this medium was to show whether or not *S. oreophila* could survive without B vitamins and glycine without any other parameters being altered. Plants were expected to have no problem with this medium because plants are capable of synthesizing these things on their own (1995; Roje 2007). Since this was a control to see how plants would handle the absence of these nutrients, only ten plants were grown on this medium. A set of inoculated plants was unnecessary.

Medium 2703 is the control medium (2493) with citrate used as an iron chelator in place of EDTA. The purpose of this medium was to show whether or not switching chelators would have a negative effect on plant growth in the absence of other parameters being altered. Only ten plants were grown on this medium. A set of inoculated plants was unnecessary. The concentration of citrate used was based on the proportion of iron to citrate in a recipe from Kvaalen *et al.* (Kvaalen, Daehlen et al. 2005).

Medium 2700 is the control medium (2493) without the sucrose and without all forms of nitrogen besides EDTA. The purpose of this medium was to see how the plants fair without any nitrogen besides EDTA. This medium was made because we were afraid that the plants would do very poorly without EDTA.

Medium 2701 is the true nitrogen-free medium. It is the control medium (2493) without sucrose and without all forms of nitrogen. This is the medium on which we hoped to see a difference between inoculated plants and clean plants.

This experiment also included many bacteriological media. See Table 2 for details and recipes.

Table 1: List of Pitcher Plant Media

Medium	Recipe (per Liter)	Purpose
2517	2493 + 2.0mg trans-zeatin(Northcutt, Davies et al. 2009)	Asexual multiplication of pitcher plants
2493*	0.66mg glycine, 30g sucrose, 0.1g myo-inositol, 1.23mg Na ₂ EDTA·2H ₂ O, 9.17mg FeSO ₄ ·7H ₂ O, 0.17mg nicotinic acid (vitamin B3), 0.17mg pyridoxine HCL (vitamin B6), 0.33mg thiamine HCL (vitamin B1), 2.05mg H ₃ BO ₃ , 2.84mg ZnSO ₄ ·7H ₂ O, 5.57mg MnSO ₄ ·H ₂ O, 0.083mg Na ₂ MoO ₄ ·2H ₂ O, 0.275mg KI, 0.008mg CuSO ₄ ·5H ₂ O, 0.008mg CoCl ₂ ·6H ₂ O	Vigorous growth of pitcher plants, control medium
2566	2493 – NH ₄ NO ₃ – KNO ₃ + K ₂ SO ₄ (potassium substitute)(Bucalo 2009)	Medium eliminated from older version of study
2567	2566 – sucrose(Bucalo 2009)	Low-nitrogen medium
2633	2493 – sucrose	show that pitcher plants can grow without sugar
2700	2493 – sucrose – all sources of nitrogen besides EDTA	Determine whether plants need EDTA provided
2701	2493 – sucrose – all sources of nitrogen + citrate	Determine the effect of total absence of nitrogen
2702	2493 – B vitamins - glycine	Show that plants can grow without B vitamins and glycine
2703	2493 – EDTA + citrate	Determine effect of substituting citrate for EDTA

* In the 1980s, the Atlanta Botanical Gardens developed this medium based on Murashige and Skoog's MS medium (Murashige and Skoog 1962). It is closer to 0.38 in dilution and has proportionally less CaCl₂·2H₂O and MgSO₄·7H₂O. This may have been done because too much calcium or manganese can be toxic to these plants (Adamec 1997).

Table 2: List of Bacteriological Media

Medium	Recipe (per Liter)	Purpose
BLK	Per liter: 7g Na ₂ HPO ₄ · 12H ₂ O; 1g KH ₂ PO ₄ ; 10mg CaCl ₂ · 2H ₂ O; 2mg ferric citrate; 20mg MgSO ₄ · 7H ₂ O; 20mM succinate(Bruhn, Lenke et al. 1987). C-Mix was also added so that succinate would not be the only carbon source.	Nitrogen-free medium for growing nitrogen-fixing bacteria
BLKN	BLK + 0.5g ammonium chloride per liter	A nitrogen-containing comparison to BLK
C-Mix	Per liter: 0.3543g succinate, 0.52g glucose, 0.32g lactate, 0.66g gluconate, and 0.54g fructose(Taghavi, Garafola et al. 2009).	The mix of carbon sources added to BLK and BLKN
LB	LB Agar (Sigma Life Science, L7025-500TAB)	For growing nonfastidious bacteria
Indexing Medium	Bacto® Nutrient Broth (Difco, 0003-17-8)	For determining presence or absence of bacteria

2.4 Re-isolation

In order to determine that the results of this experiment are valid, it is important to show that the *Burkholderia* have endophytic competence and did in fact end up inside the *S. oreophila* plants.

After the experiment had been running for three months and the plants were no longer needed, two inoculated plants from media 2700, 2701, 2633, and 2493 were selected. Media 2702 and 2703 were not included because none of the plants growing on these media were inoculated with bacteria. Medium 2567 was not included because it was not an important part of the experiment after the media were redesigned.

The procedure used was very similar to that used by Stoltzfus *et al.* (Stoltzfus, So et al. 1997). Selected plants were surface sterilized using the before-mentioned hydrogen peroxide procedure (MacKay, Becwar et al. 2006). 200µl of water from the last wash was spread on LB plates to ensure that the sterilization treatment was successful. The media from the boxes the plants were growing in was checked for bacteria (as described in Section 2.2) to ensure that the bacteria were still alive. Surface-sterilized plants were dissected on sterile petri dishes with sterile scalpels and tweezers. Portions

of the leaves and roots were kept separate and weighed in pre-weighed sterile petri dishes. Wet weights were recorded.

Leaves and roots were transferred to sterile 100ml stainless steel Waring commercial laboratory blender cups along with 30ml sterile deionized water. Samples were macerated on the low setting for one minute each.

200 μ l of macerate was spread on three plates of BLK and three plates of BLKN. One milliliter of macerate was added to 9ml of sterile deionized water to make a 1:10 dilution. 200 μ l of this dilution was spread on three plates of BLK and three plates of BLKN. Thus, for each plant, there were three plates of BLK and three plate of BLKN for 1:1 and 1:10 dilutions for both leaves and roots. This adds up to a total of 24 plates per plant. All BLK and BLKN batches included C-Mix (see Table 2) as a carbon source.

After some deliberation, it was decided that the crowns of the plants should be tested, as well. The crown is identified as the “persistent base of an herbaceous perennial” (Harris and Harris 2001). It is where the roots and leaves both grow from. Two more inoculated plants from each of the selected media were given the same treatment as mentioned above. Only instead of macerating the leaves and roots, the crowns were macerated.

All plates were left to grow at 25-26 °C in a cabinet for one week before colonies were counted.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 The Effect of Medium Content on Plant Biomass

Percent growth was calculated from the biomass data collected in order to account for initial differences in mass between plants. The plants were transferred once per month, but the data was converted into percent growth per day because the plants were not always transferred exactly 30 days apart.

Plants growing on the control medium without sugar (2633) performed significantly worse than those with sugar (2493, 2702, and 2703) (P-value: 0.0005). See Figure 4. The analysis is a one-way ANOVA, done to determine whether plants grown without EDTA (2703) or without B vitamins and glycine (2702) could perform as well as the control medium.

Plants on the control media with sugar (2493, 2702, and 2703) had a significantly higher rate of percent biomass produced per day compared to all other treatments (P-value: 0.0000). Plants grown on medium 2700 (with EDTA) produced more biomass per day than plants grown on 2701 (citrate instead of EDTA). The P-value is 0.0160. See Figure 5.

3.2 The Effect of *Burkholderia* Inoculation on Plant Biomass

Plants inoculated with *Burkholderia* on medium 2493 (full nitrogen and full sugar) produced less biomass than plants that were not inoculated (P-value: 0.0968). See Figure 6. Plants inoculated with *Burkholderia* on 2633 (full nitrogen and no sugar) produced more biomass than plants that were not inoculated (P-value: 0.0598). See Figure 7. Plants inoculated with *Burkholderia* on 2567 (low nitrogen and no sugar) produced more biomass than plants that were not inoculated (P-value: 0.0800). See Figure 8. Plants inoculated with *Burkholderia* on 2700 (EDTA is only nitrogen-containing compound, no sugar) produced less biomass than plants that were not inoculated (P-value: 0.0576). See Figure 9. Plants inoculated with *Burkholderia* on 2701 (no nitrogen,

no sugar) produced less biomass than plants that were not inoculated, but the P-value was only 0.3963. See Figure 10. Most of the experiments were significant at a 10% confidence level.

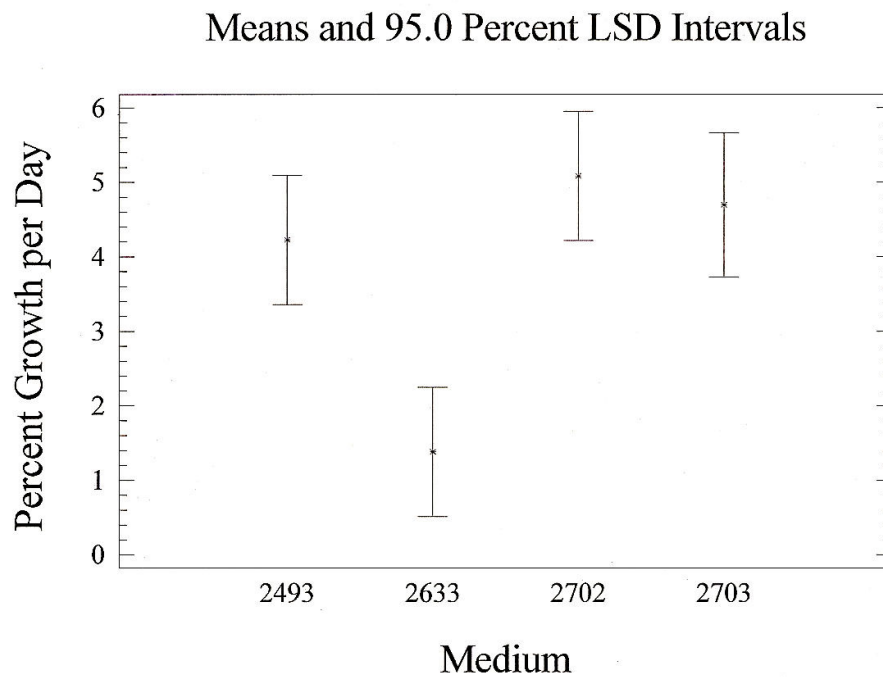


Figure 4: One-Way ANOVA Graph of Percent Growth per Day (grams) on Control Media. The media with sugar (2493, 2702, and 2703) produced significantly more biomass than the medium without sugar (2633). Media 2493, 2702, and 2703 were not statistically different from each other (P-value: 0.0005).

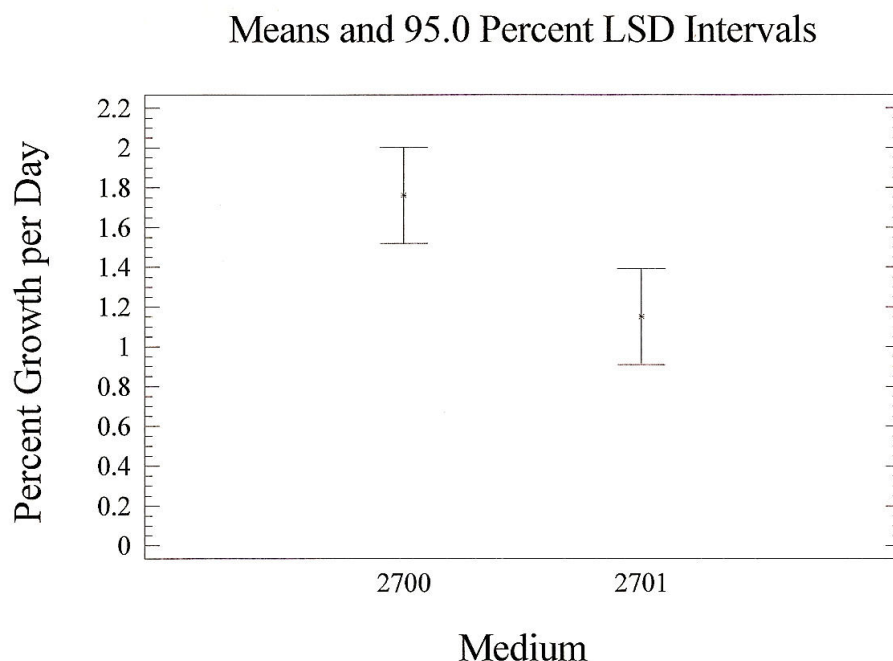


Figure 5: One-way ANOVA Graph Comparing Percent Biomass Produced Per Day (grams) While Growing on Media 2700 or 2701. Medium 2700 is nitrogen-free except for EDTA. Medium 2701 is completely nitrogen-free. Plants grown on medium 2700 produced significantly more biomass per day than plants grown on 2701 (P-value: 0.0160).

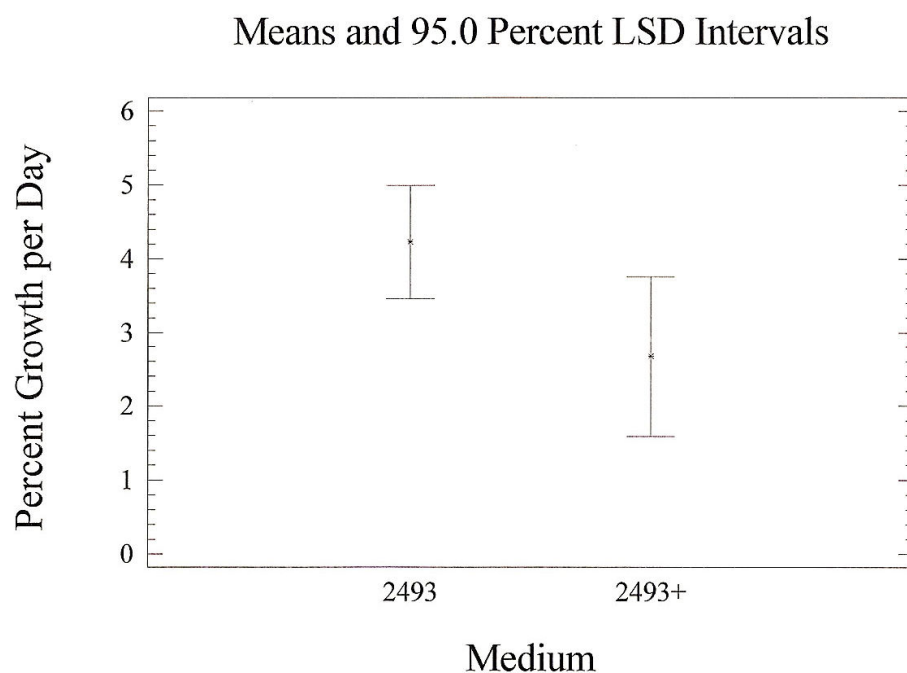


Figure 6: One-way ANOVA Graph Comparing Percent Biomass Produced Per Day (grams) by *S. oreophila* Growing on Medium 2493 With and Without *Burkholderia*. The plus sign indicates *Burkholderia*. Inoculated plants produced less biomass than plants that were not inoculated (P-value: 0.0968).

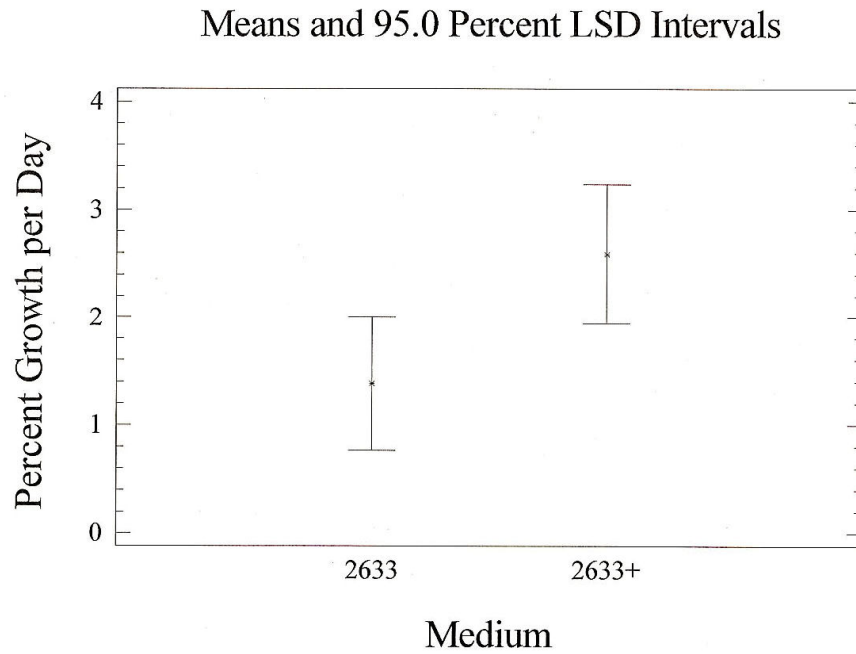


Figure 7: One-way ANOVA Graph Comparing Percent Biomass Produced Per Day (grams) by *S. oreophila* Growing on Medium 2633 With and Without *Burkholderia*. The plus sign indicates *Burkholderia*. Inoculated plants produced more biomass than plants that were not inoculated (P-value: 0.0598).

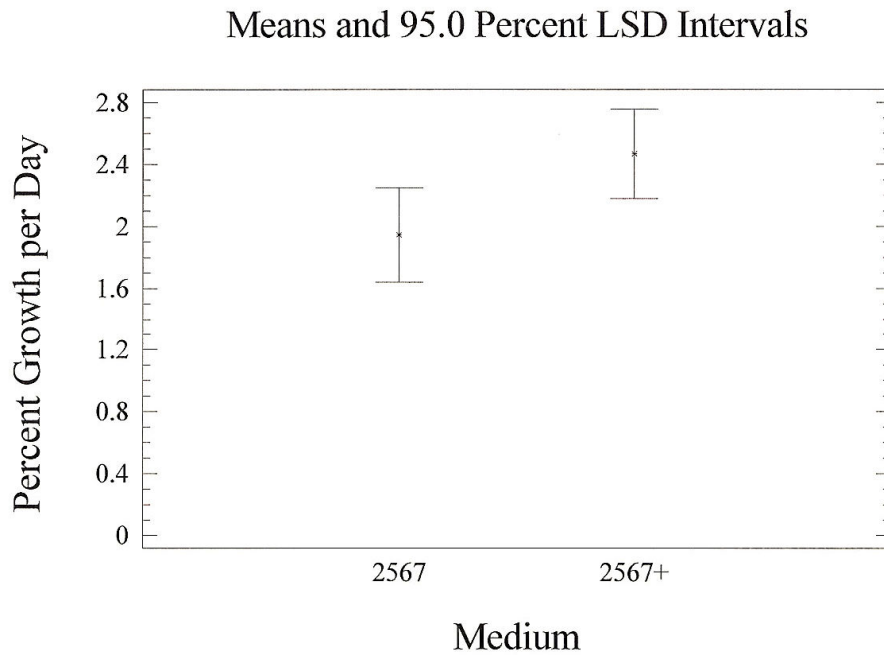


Figure 8: One-way ANOVA Graph Comparing Percent Biomass Produced Per Day (grams) by *S. oreophila* Growing on Medium 2567 With and Without *Burkholderia*. The plus sign indicates *Burkholderia*. Inoculated plants produced more biomass than plants that were not inoculated (P-value: 0.0800).

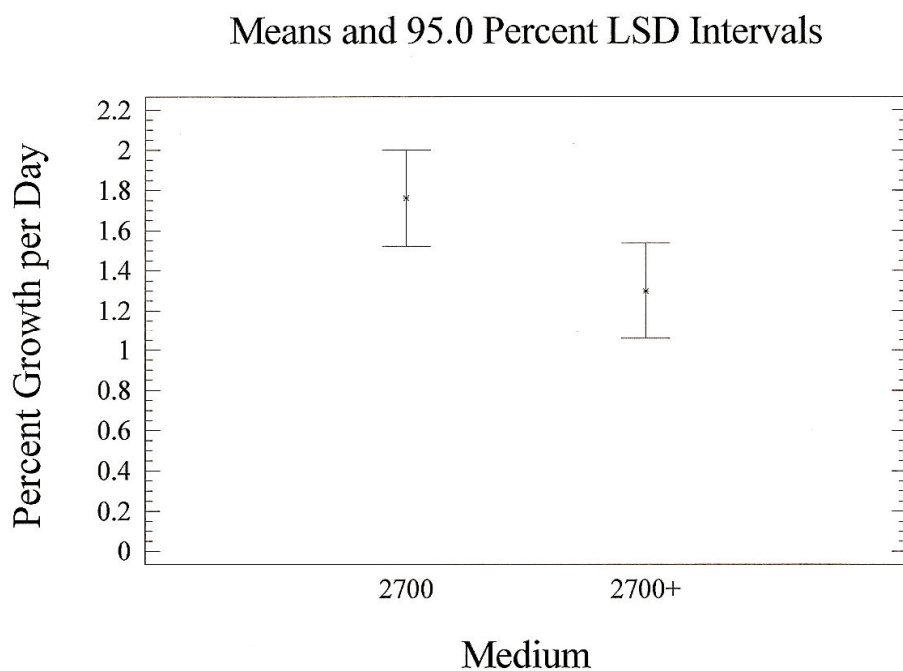


Figure 9: One-way ANOVA Graph Comparing Percent Biomass Produced Per Day (grams) by *S. oreophila* Growing on Medium 2700 With and Without *Burkholderia*. The plus sign indicates *Burkholderia*. Inoculated plants produced less biomass than plants that were not inoculated (P-value: 0.0576).

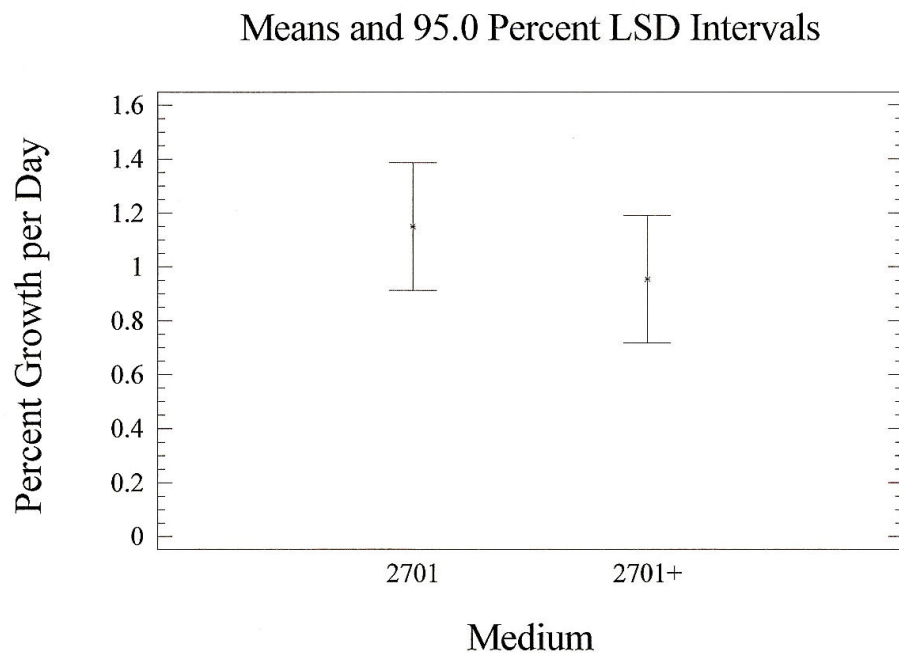


Figure 10: One-way ANOVA Graph Comparing Percent Biomass Produced Per Day (grams) by *S. oreophila* Growing on Medium 2701 With and Without *Burkholderia*. The plus sign indicates *Burkholderia*. Inoculated plants produced less biomass than plants that were not inoculated (P-value: 0.3963).

3.3 The Effect of Medium Content on Root Growth

Growth per day was measured instead of percent growth per day because all of the plants started with 0cm of roots. Analyses were based on total root length each plant produced (the sum of the lengths of all roots on a plant).

None of the plants growing on the control media (2493, 2633, 2702, 2703) produced significantly more root length than the others (P-value: 0.4052) when not inoculated (Figure 11). The analysis is a one-way ANOVA, done to determine whether plants grown without sugar (2633), without EDTA (2703), or without B vitamins and glycine (2702) could perform as well as the control medium.

Plants on the control media (2493, 2633, 2702, and 2703) without bacteria did significantly better than all of the others (P-value: 0.000). Plants grown on 2700 (with EDTA) and 2701 (citrate instead of EDTA) did not produce significantly different root length (P-value: 0.9677). See Figure 12.

3.4 The Effect *Burkholderia* Inoculation on Root Growth

Plants inoculated with *Burkholderia* on medium 2493 (full nitrogen and full sugar) produced less total root length than plants that were not inoculated (P-value: 0.0109). See Figure 13. This matches the biomass data.

Plants inoculated with *Burkholderia* on 2633 (full nitrogen and no sugar) produced less total root length than plants that were not inoculated (P-value: 0.0007). See Figure 14. This does not match the biomass data.

Plants inoculated with *Burkholderia* on 2567 (low nitrogen and no sugar) produced more total root length than plants that were not inoculated, but the P-value is only 0.6342. See Figure 15. This matches the biomass data.

Plants inoculated with *Burkholderia* on 2700 (EDTA is only nitrogen-containing compound, no sugar) produced more total root length than plants that were not inoculated (P-value: 0.4087). See Figure 16. This does not match the biomass data.

Plants inoculated with *Burkholderia* on 2701 (no nitrogen, no sugar) produced more total root length than plants that were not inoculated, but the P-value was only 0.7413. See Figure 17. This matches the biomass data.

Most of the experiments were not statistically significant, even to the 10% confidence level.

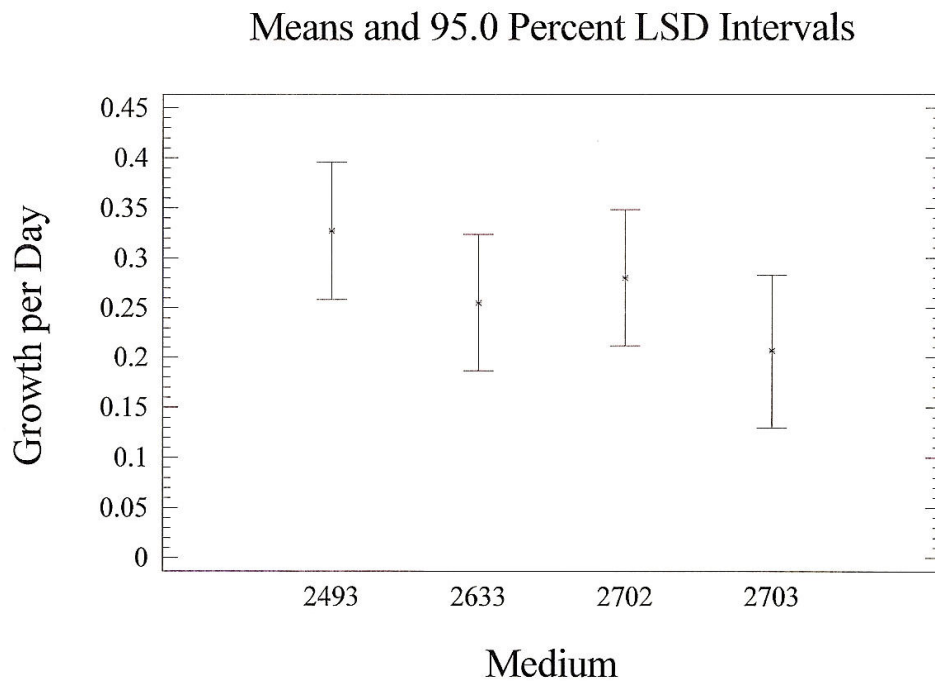


Figure 11: One-way ANOVA Graph of Root Growth Per Day (cm) on Control Media. None of the control media were significantly different from the others (P-value: 0.4052).

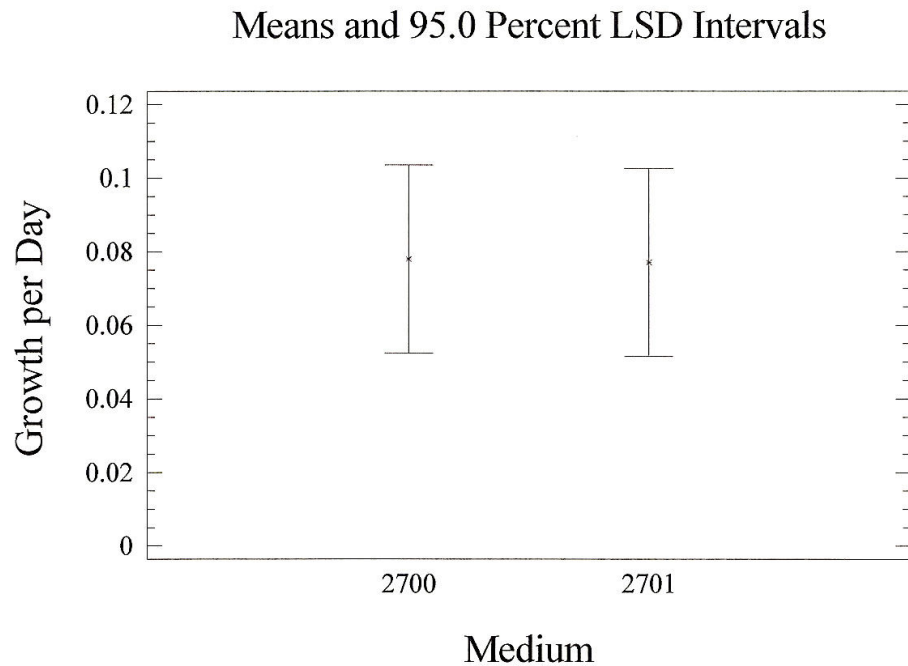


Figure 12: One-Way ANOVA Graph Comparing Root Growth per Day (cm) of Plants Growing on Medium 2700 or 2701. There was no significant difference in root production between plants on these two media (P-value: 0.9677).

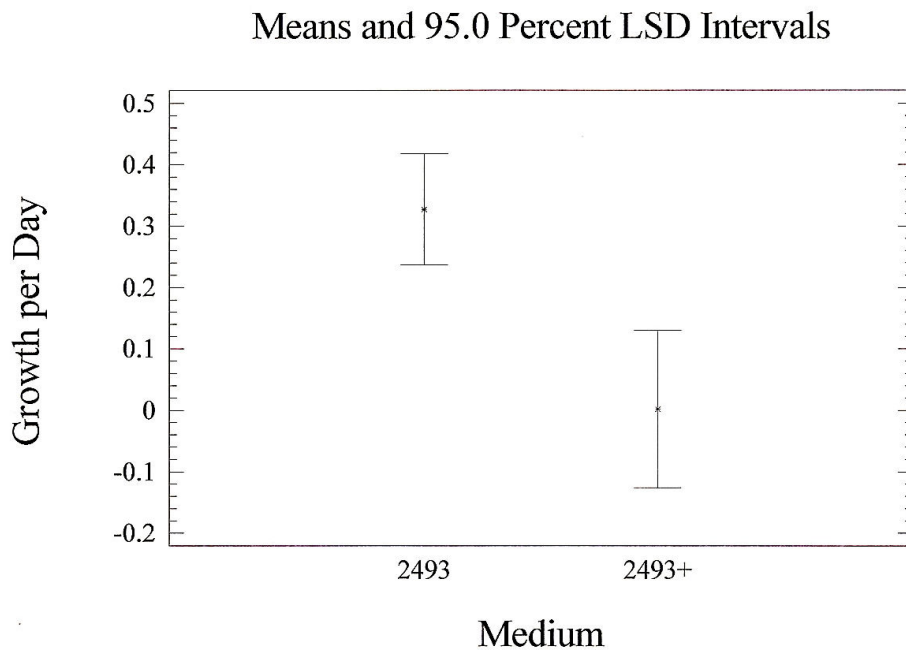


Figure 13: One-way ANOVA Graph Comparing Root Growth Per Day (grams) by *S. oreophila* Growing on Medium 2493 With and Without *Burkholderia*. The plus sign indicates *Burkholderia*. Inoculated plants produced less total root length than plants that were not inoculated (P-value: 0.0109).

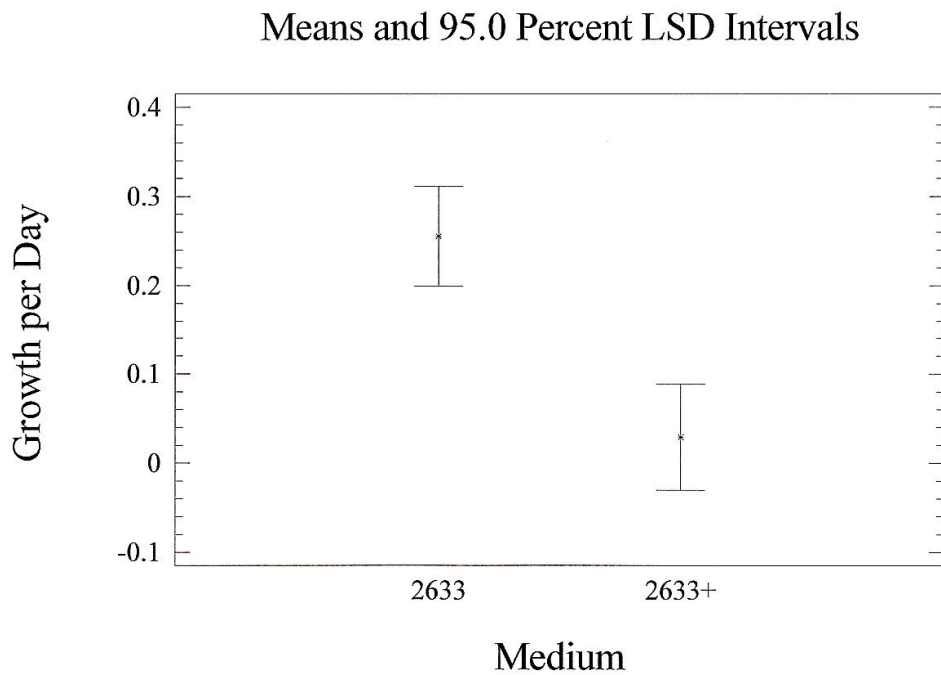


Figure 14: One-way ANOVA Graph Comparing Root Growth Per Day (grams) by *S. oreophila* Growing on Medium 2633 With and Without *Burkholderia*. The plus sign indicates *Burkholderia*. Inoculated plants produced less total root length than plants that were not inoculated (P-value: 0.0007).

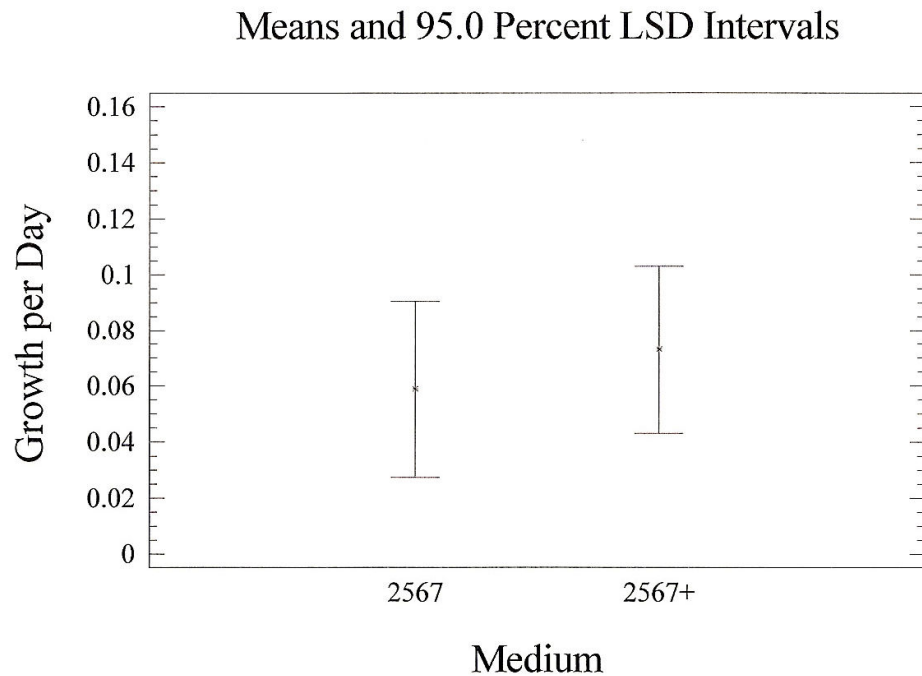


Figure 15: One-way ANOVA Graph Comparing Root Growth Per Day (grams) by *S. oreophila* Growing on Medium 2567 With and Without *Burkholderia*. The plus sign indicates *Burkholderia*. Inoculated plants produced more total root length than plants that were not inoculated (P-value: 0.6342).

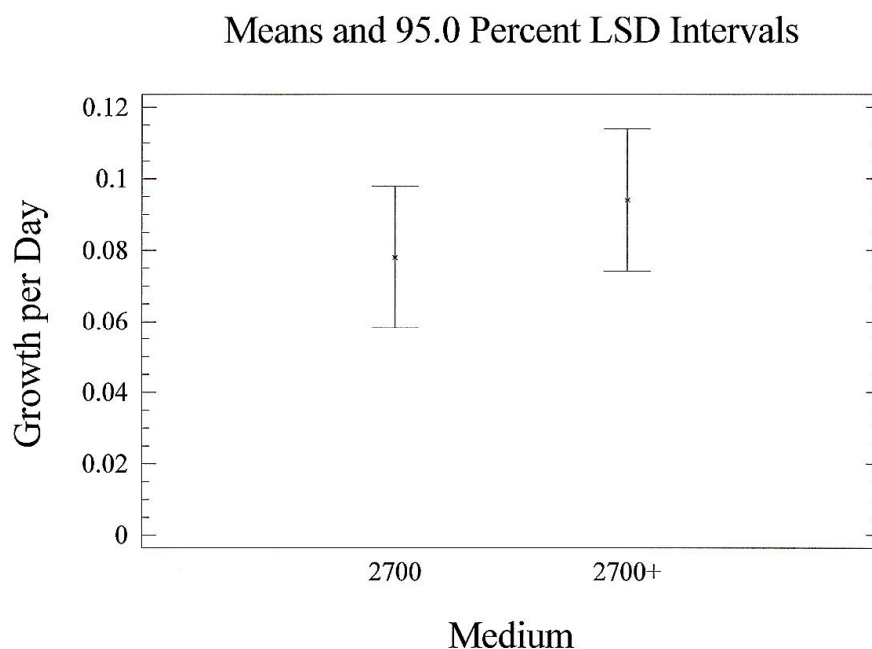


Figure 16: One-way ANOVA Graph Comparing Root Growth Per Day (grams) by *S. oreophila* Growing on Medium 2700 With and Without *Burkholderia*. The plus sign indicates *Burkholderia*. Inoculated plants produced more total root length than plants that were not inoculated (P-value: 0.4087).

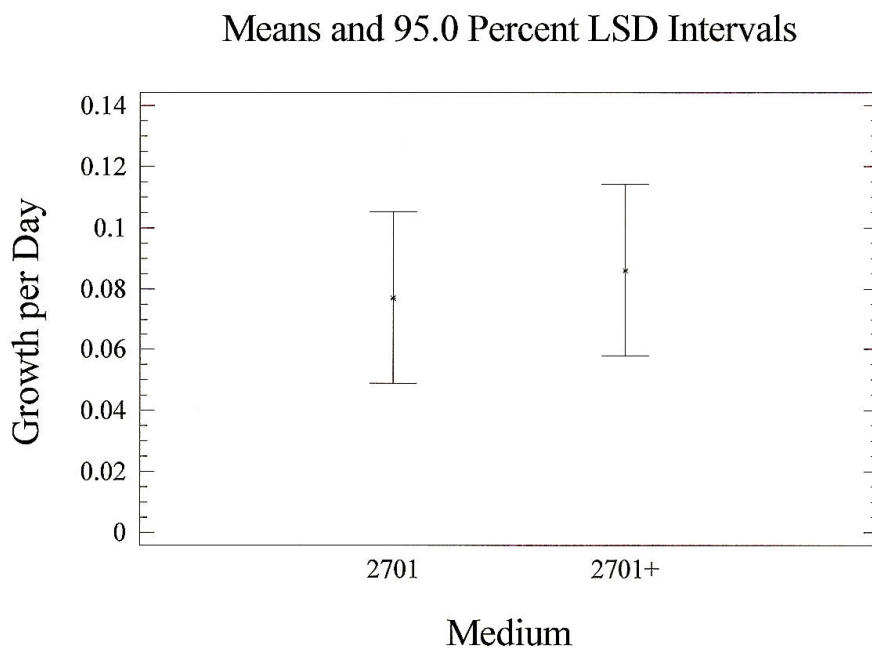


Figure 17: One-way ANOVA Graph Comparing Root Growth Per Day (grams) by *S. oreophila* Growing on Medium 2701 With and Without *Burkholderia*. The plus sign indicates *Burkholderia*. Inoculated plants produced more total root length than plants that were not inoculated (P-value: 0.7413).

3.5 Re-isolation

Table 3: Colony Forming Units isolated from different plant parts on different media.

Medium	Part	CFU g ⁻¹ BLK	CFU g ⁻¹ BLKN
2701	Leaf	1.6×10^3	3.4×10^2
	Root	5.6×10^5	3.9×10^5
	Crown	8.3×10^5	4.0×10^5
2700	Leaf	0	0
	Root	0	0
	Crown	0	0
2633	Leaf	0	0
	Root	0	0
	Crown	0	0
2493	Leaf	3.3×10^5	1.8×10^5
	Crown	7.5×10^6	9.4×10^6

No colonies were isolated from the leaves, roots, or crowns of inoculated plants grown on media 2700 (nitrogen-free except for EDTA) or 2633 (control medium without sugar). See Table 3.

Many colonies were isolated from the leaves, roots, and crowns of inoculated plants grown on 2701. The amount of bacteria was two to three orders of magnitude greater in the roots and crowns than it was in the leaves.

Many colonies were isolated from the leaves and crowns of plants grown on 2493. The amount of bacteria found in the crown was one magnitude greater than the amount found in the leaves.

No colonies were isolated from the roots of inoculated plants grown on 2493, because none of those plants grew any roots during the experiment. All other plants grew roots, including plants grown on 2493 that were not inoculated with bacteria.

More colonies were isolated from the plants grown on 2493 than from the plants grown on 2701.

The media from the boxes the plants were growing in were all checked for bacteria on the same day the re-isolation was carried out. All of the results were positive, meaning that there was definitely *Burkholderia* in the media. All of the bacteria isolated from the plants had uniform morphology that matched that of the *Burkholderia* the plants were inoculated with. One sample of the bacteria that was re-isolated from a plant was submitted for 16S rRNA sequencing, verifying that the bacteria that came out of the plants was indeed the *Burkholderia* that the plants were inoculated with.

3.4 Discussion

Plants Grow Well Without B Vitamins, Glycine, or EDTA, but not Without all Three

Since there was no significant difference between the control media with sugar (2493, 2702 and 2703), we know that *S. oreophila* can survive well without being provided with B vitamins, glycine, or EDTA (Figure 4). It was expected that removing B vitamins and glycine would not be an issue, because plants are capable of synthesizing these compounds (Roje 2007).

There is no literature that involves using citrate as an iron-chelator in pitcher plants, but there is data involving citrus trees that cites EDTA as the best chelator by far (Leonard and Stewart 1952). Therefore, we expected the plants on 2703 to grow less than the plants on 2493, but the results suggest that citrate worked just as well. A plant can get sufficient iron without the addition of EDTA as long as the concentration of iron is high enough (Leonard and Stewart 1952). Also, pitcher plants are known to be very efficient in nutrient-poor habitats (Juniper, Robins et al. 1989). Perhaps the reason that plants on 2703 grew as well as plants on 2493 is that these media both contained more than enough iron for these very efficient plants.

However, plants grown on medium 2701 (nitrogen-free) produced significantly less biomass than plants grown on 2700 (nitrogen-free except for EDTA) (P-value:

0.0160). See Figure 5. Since plants grew just as well on 2703 when compared to 2493, we expected similar results when comparing 2700 and 2701. Maybe there is a threshold for nitrogen-containing compounds that 2701 dipped below since it contains no nitrogen at all.

Plants growing on the control medium without sugar (2633) performed significantly worse than those with sugar (2493, 2702, and 2703) (P-value: 0.0005). It is not surprising that plants without sugar grow less than plants provided with sugar. This comparison was done to show that there is a decrease in biomass production when no sugar is added to the media, but the plants can still grow. In previous experiments (Bucalo 2009), bacteria seemed to choke out the plants when provided with sugar. For this experiment, we opted to remove the sugar from the media. Leaving sugar in the media would have the potential to skew the results, because sugar is theoretically what the *Burkholderia* would be gaining from the plant if a symbiotic relationship occurs. Therefore, we want any sugar that the *Burkholderia* is benefitting from to come from the plant.

Burkholderia Aids Biomass Production when Nitrogen is Low, but not When Nitrogen is Absent

Most of the comparisons of inoculated plants versus non-inoculated plants on individual media become statistically significant at 10% uncertainty (P-value less than 0.10) instead of 5% uncertainty (P-value less than 0.05). The *Burkholderia* hinders growth on 2493 and 2700 (P-values of 0.0968 and 0.0576 respectively). For the plants on medium 2493, this may be because the *Burkholderia* choked the plants, utilizing too many of the nutrients in the medium to the detriment of the plants (see Figures 13 and 18). For the plants growing on medium 2700, the reason that the *Burkholderia* did not help may be due to very low level of nitrogen. We may have made the nitrogen content of the medium so low (almost none, just EDTA) that the plants were not getting enough

nitrogen even with the help of the *Burkholderia*. The *Burkholderia* may have utilized enough of the scant nutrients in the medium that the plant actually suffered.

The *Burkholderia* aids growth on 2633 and 2567 (P-values of 0.0598 and 0.080 respectively). The amount of nitrogen in these plants was kept low, but not totally removed. As suggested above, there may be a threshold for how much nitrogen can be removed before the *Burkholderia-Sarracenia* relationship actually becomes detrimental. These media provide an amount that is above the threshold. It is also possible that when *Burkholderia* are provided with more nitrogen, they are able to fix more nitrogen, providing a greater benefit to the plant. It is also possible that the plants need some nitrogen in order to produce enough sugar to initially tempt the *Burkholderia* into symbiosis.

There is no difference on 2701 (P-value: 0.3963). Like medium 2700, this medium may be below a nitrogen threshold. Like on medium 2700, the inoculated plants produced less biomass than the ones that were not inoculated, but the P-value was high (0.3963). Still, the P-values in these experiments were low enough to suggest that the results may be more statistically significant if the sample size had been larger. Alternatively, there may be more to the story than our tests can elucidate. For example, *Burkholderia* may work in conjunction with other endophytes, or its main growth-promoting influence may be antagonism against a pathogen that was not present during the experiment.

Roots Grow Best In Presence of Nitrogen

Plants on all of the high-nitrogen control media (2493, 2702, 2703, and 2633) without bacteria grew significantly more root length than all other treatments. None of the control media performed any better than either of the other control media. It is surprising that the control media containing sugar (2493, 2702, and 2703) did not outperform the

control medium without sugar (2633) as they did when comparing biomass. It was expected that plants without sugar would not grow as well as plants with sugar. However, while the plants with sugar did not grow more roots, they did have a greater increase in biomass. This means that the majority of that added biomass was due to leaf production. Maybe the nutrient that the plants needed more of was sugar, and they produced more leaves when able in order to achieve that goal. Pitcher plants are adapted to nutrient-poor soil, so the media that was provided to them may have been more than enough to supply the necessary minerals (Juniper, Robins et al. 1989). Therefore, instead of using energy to make more roots, the plants could focus on increasing photosynthetic output instead.

Plants grown on media 2700 produced more biomass per day than plants grown on medium 2701, but did not produce a statistically different amounts of roots (Figures 5 and 12). This is more evidence that citrate may be a viable replacement for EDTA in pitcher plants, but we may not have used enough.

Roots Grow Best in Absence of Burkholderia

Plants on the full-nitrogen control media 2493 and 2633 grew better in the absence of the *Burkholderia* (P-values: 0.0109 and 0.0007 respectively). The bacteria actually seemed to choke out the plants on 2493 such that they did not produce roots at all (Figures 13 and 18). However, the bacteria did not kill the plants, because they still produced biomass. All other media (with low nitrogen or no nitrogen) showed no significant difference in root production. This means the bacteria most likely were not producing any exudates that the plant could use outside of the plants, because plants are actually known to produce excess roots in order to fully utilize patches of high nutritional value (McNickle and Cahill 2009).

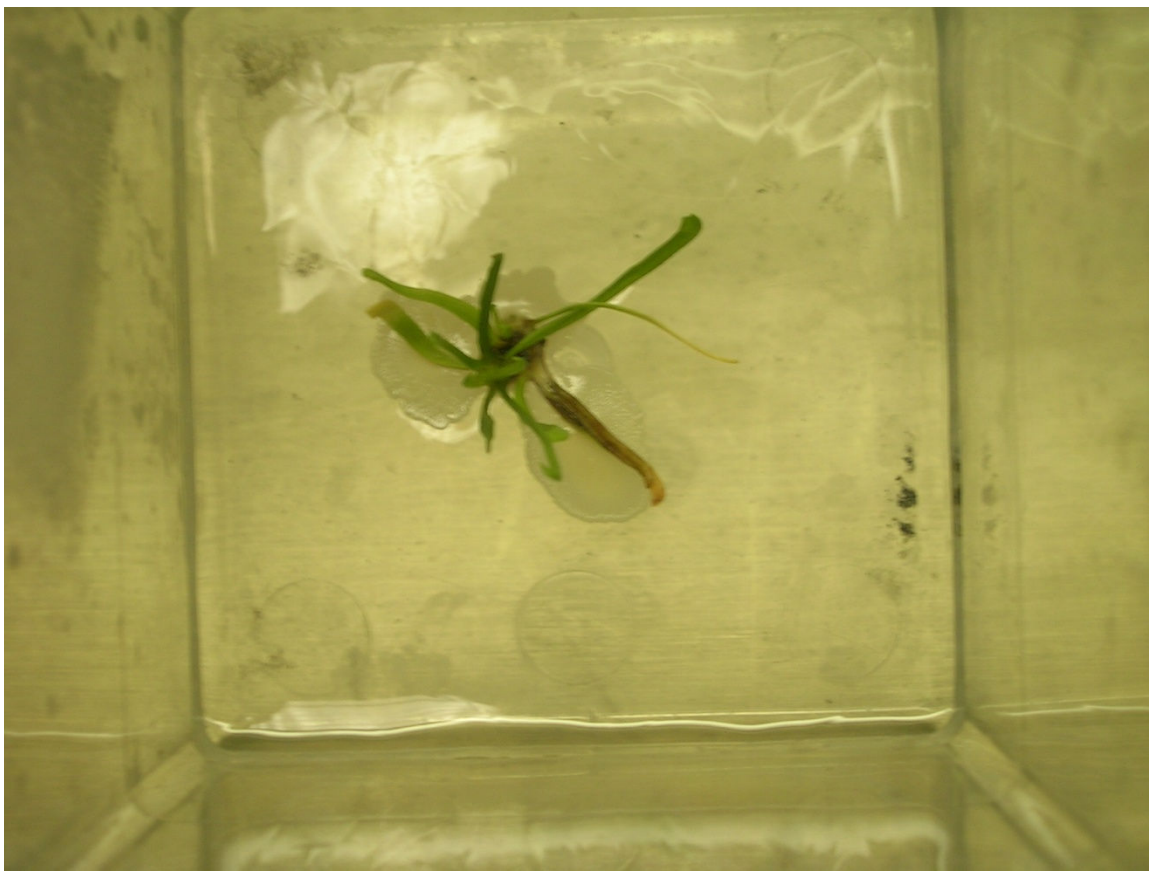


Figure 18: An *S. oreophila* plant growing on medium 2491 surrounded by *Burkholderia*.

Re-isolation Confirmed Plant-Colonization on Some Media but Not All

No colonies were isolated from the leaves or roots of plants grown on media 2700 (nitrogen-free except for EDTA) or 2633 (control medium without sugar). See Table 3. It is possible that the results from plants grown on medium 2633 are spurious, because they were older than the other plants at the time of the experiment and were visibly declining in health. It is unclear why the *Burkholderia* should have gotten into plants grown on 2701 but not plants grown on 2700. However, since *Burkholderia* was recovered from the nitrogen-free medium (2701), the hypothesis that there may be a nitrogen threshold for the *Burkholderia-Sarracenia* relationship is still viable.

It is also worth noting that all of the boxes that the plants for this experiment were grown in were checked for presence of *Burkholderia* (as in section 2.2), and all of the

results were positive. So, the *Burkholderia* did survive through the experiment and was transferred along with the plants every month, but somehow did not get into the plant.

Many colonies were isolated from the leaves and crowns of plants grown on 2701 (true nitrogen-free medium) and 2493 (control medium) and the roots of plants grown on 2701. More CFU were found in the roots and crowns of 2701 than in the leaves. This is expected because the roots are where we would expect nitrogen-fixing bacteria to confer the most benefit as the roots are the primary gatherers of nutrients. The leaves of plants grown on 2493 contained more bacteria than the leaves of 2701 but less than the roots of 2701. The crowns of plants grown on 2493 had more CFU than any other group. It is not surprising that the plants on 2493 had more bacteria in them than the plants on 2701 because medium 2493 is very hospitable to the growth of bacteria, as it contains sugar.

No colonies were isolated from the roots of inoculated plants grown on 2493, because none of those plants grew any roots during the experiment. This was a surprise. Perhaps the hospitable sugary medium helped the *Burkholderia* grow so much that it made the environment unfavorable for the roots. However, the plants were still able to grow.

Did the plants that actually welcomed bacteria produce more biomass than those that did not? No, plants on 2493 showed a statistically significant negative correlation at a 10% confidence interval, and plants grown on 2701 showed no significant difference. This might imply that the *Burkholderia* isolated isn't a helpful bacterium at all, but a pathogen. Alternatively, the *Burkholderia* might confer a benefit that we were not able to witness due to the parameters of our experiment. For example, the *Burkholderia* may control pathogens in the wild. The plants in this experiment were sterile until the *Burkholderia* was added. The *Burkholderia* was the only microbe introduced.

In retrospect, plants that were grown on medium 2567 should have been part of the re-isolation experiment, as well. These plants were left out because, at the time, this low-nitrogen medium was thought to be an irrelevant mistake made during the experimental process. However, the results this medium yielded turned out to be interesting in the end.

A possible snag in the experimental design may be the amount of light the plants were grown in. The amount of light the plants received was probably lower than the bright sunlight they would get in the wild. Without adequate sunlight, the plants may not have been producing enough sugar to tempt the *Burkholderia* into symbiosis.

CHAPTER 4: CONCLUSIONS

4.1 Summary of Experimental Conclusions

In this work, we studied the following:

- *Burkholderia*'s effect on the biomass of *S. oreophila*
- *Burkholderia*'s effect on the root growth of *S. oreophila*

The *Burkholderia* that was isolated from *S. oreophila* during a previous experiment was shown to have mixed effects on plant biomass production and negative effects on plant root production. Since *Burkholderia* had a positive effect on plants growing on low-nitrogen media and a negative effect on plants grown on the nitrogen-free medium, the results suggest that there is a nitrogen threshold that needs to be met by the environment before the *Burkholderia-Sarracenia* symbiosis can occur.

The root length experiment suggests that roots grow best when nitrogen is present and *Burkholderia* is absent. It makes sense that the roots grow better in full-nitrogen, because plants produce more roots in order to fully utilize nutrient-rich pockets (McNickle and Cahill 2009). The *Burkholderia* may have hindered root growth by competing for these nutrients. Plants have also been known to produce excess roots at the expense of the shoots in order to search for nutrients in a low nutrient environment, but there is a point at which nutrients are so low that more roots would not be able to grow (Hussner 2010). In this experiment, the media with high levels of nitrogen were rich enough to stimulate much growth. The media with low levels of nitrogen were enough to prevent the search response from happening.

4.2 Future Work

In order to test the new hypothesis that there is a nitrogen-threshold that needs to be reached before the *Burkholderia-Sarracenia* symbiosis can occur, *Sarracenia*

should be grown on media differing only in nitrogen content. Hopefully we would see that as nitrogen level dips below the threshold, the *Burkholderia* switch from being helpful to being a hindrance. The re-isolation experiment should be done for inoculated plants on all of these media.

Since the plants growing on medium 2701 produced less biomass than plants growing on 2700, future nitrogen-free media should contain greater amounts of citrate. The paper that was used to calculate the proportion of citrate to iron was about subalpine fir (Kvaalen, Daehlen et al. 2005). Maybe fir trees need less help from iron-chelators than pitcher plants, or maybe the medium was never optimal for either plant.

There could be another reason that the plants on 2701 produced less biomass than the plants on 2700. Late in the experiment, we realized that citrate can serve not only as an iron-chelator, but also as a carbon source for bacteria. Some species of *Burkholderia* have been shown to be capable of utilizing citrate as a sole carbon source (Marin, Smits et al. 2001). If the *Burkholderia* used in this experiment can utilize citrate as a lone carbon source, the plants on 2701 may become overrun with bacteria like the plants on 2493 (with sugar as a carbon source) did. The *Burkholderia* should be streaked on citrate agar (a medium containing citrate that turns bright blue when citrate is utilized) to determine whether it can utilize citrate.

EDTA can also be used as a carbon and nitrogen source by bacteria, but bacteria cannot degrade the EDTA in the presence of a molar equivalent of iron (Nortemann 1992). If citrate works the same way, it would not be a useable carbon source and would not be problematic in the experiment. Iron could be added to the citrate agar to determine whether this is the case or not.

The sample size should also be increased. Some of the biomass data was very close to statistical significance, implying that a larger sample size might push the data into the realm of significance.

Future work could also include investigating other possible function the *Burkholderia* may play. It may confer resistance to a bacterial or fungal pathogen. To determine whether or not this is the case, wild populations should be checked for potentially pathogenic microbes. These microbes could be isolated and pitted against the *Burkholderia* in the lab.

We would also like to investigate whether there are other endophytes within *S. oreophila* and in what relative quantities. We would like to isolate bacteria from the rhizosphere (soil near roots), endorhizosphere (root tissue), phyllosphere (surface of leaves), and endophyllosphere (leaf tissue) as done with *Drosera* by Albino *et al.* (Albino, Saridakis *et al.* 2006). We would also like to compare different *Sarracenia* species across different geographical regions.

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